New strategies to increase efficacy of vaccines for tetanus-diphtheria-pertussis (TDaP) and other targets

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Abstract

A vaccine is a biological preparation that actively stimulates adaptive immunity to protect against a target disease. Prophylactic vaccination is the most effective method of preventing infectious diseases and may be applied to the prevention or treatment of other non-communicable diseases (e.g. Cancer, Alzheimer's). Herd immunity due to vaccination is largely responsible for the worldwide eradication of smallpox and containment of other diseases such as polio (type 2), measles, tetanus, and pertussis (whooping cough) [1]. Traditional vaccines consist of live-attenuated forms (e.g. Bacillus Calmette–Guérin vaccine, typhoid vaccine, measles-mumps-rubella vaccines) or whole cell preparations of the target infectious agent (e.g., whole cell pertussis vaccine). Biotechnology advances have enabled newer vaccines to be based on the rational design of recombinant antigens containing highly purified components with excellent safety profiles. Conversely, the immunogenicity of such well-defined vaccine antigens may be lower compared to vaccines comprised of live attenuated or inactivated pathogen preparations. Candidate vaccines consisting of purified subunits or proteins from bacteria or viruses are less immunogenic and require the use of adjuvants to generate a strong immune response to the administered antigen. To increase vaccine efficacy, there is a need to develop more potent and safer adjuvants or immunostimulators. In my masters' project, I have explored the effects of a novel molecular adjuvant on the immune responses to a traditional tetanus-diphtheria-pertussis vaccine (TDaP).

1. Introduction-

1.1 Adjuvants

Adjuvants are defined as compounds that enhance and/or shape antigen-specific immune responses after vaccination [2]. The immune system has evolved to recognize antigenic moieties called pathogen-associated molecular patterns (PAMP). Adjuvants mimic the physical-chemical features and mechanisms of such moieties. Currently marketed adjuvants consist of mineral salts, emulsions, liposomes and virosomes, which provide a delivery system that improves antigen presentation to professional antigen presenting cells (e.g., macrophages) displaying MHC I and II receptors. Use of such adjuvants can control the release of the antigen, and modulates the quantity and quality of the immune response [2]. Another class of adjuvants includes immunostimulants, which target specific receptors or pathways involved in activation of the immune response against antigens. For example, a variety of adjuvants are ligands to PAMP called toll-like receptors (TLR). These compounds modulate cytokine production, activation of MHCI and II receptors, co-stimulatory signals involved in activation of B and T cell lymphocytes, and downstream intracellular signaling pathways [3]. Table 1 provides an overview of current adjuvants.

Table 1. Overview of approved and under development adjuvants.

Adjuvant Type	Examples	Composition	Target or formulation
Mineral salts and organic compounds	Aluminum	Aluminum phosphate and aluminum hydroxide.	TDaP, anthrax vaccine, poliomyelitis vaccine. Veterinary vaccines for avian infectious bronchitis virus, clostridium botulinum, bacteroides nodosus.
	AS03 (Adjuvant System 03)	Squalene, α-tocopherol, polysorbate 80.	Seasonal influenza virus vaccine.
	MF59	Oil-in-water squalene based	Influenza vaccine in 65^{+} years old.
	AF03	Oil-in-water squalene based	Pandemic influenza vaccine.
TLR based Adjuvants	Adjuvant System AS04	Aluminum hydroxide and monophosphoryl lipid A (MPLA, TLR4 Agonist)	Hepatitis B virus (HBV), Human papillomavirus (HPV).
	CpG	Short single-stranded synthetic DNA molecules, TLR9 ligand.	Hepatitis B virus.
	AS02*	Oil / water emulsion, of monophosphoryl lipid A (MPL) and QS21, an extract from plant Quillaria saponaria.	Malaria vaccine.
	AS15**	Liposomal formulation of TLR4 and TLR9 agonists.	Prostate cancer, Breast cancer.
	Monophosphoryl Lipid A (MPLA)	TLR4 agonist	Preclinical studies in chronic myelogenous leukemia, breast cancer vaccines.
	AS01***	Saponin QS-21 and MPLA based liposomal adjuvant	Malaria vaccine.
Particle-based Adjuvants	Virosomes	Virus derived protein based liposome	Hepatitis B virus
	Liposomes		Hepatitis A virus

* Phase 1 clinical trial for malaria concluded in 2015. Study sponsored by U.S. Army Medical Research.

* *Currently in a European Phase I/II safety and efficacy study in prostate cancer patients. Also, a Phase II trial of the recombinant Her2 protein with AS15 adjuvant is currently underway

*** On 17 November 2016, WHO has announced that malaria vaccine, RTS, S/AS01 would be rolled out in pilot projects in 3 countries in sub-Saharan Africa.

1.2 Need for newer adjuvants

Traditional adjuvants such as alum, aluminum phosphate, and aluminum hydroxide have been widely used. Although they have several advantages, they are far from ideal. Some of the limitations to alum-based adjuvants include the production of IgE antibodies leading to hypersensitivity reactions, inability to elicit cell-mediated immunity, local reactions, poor immunogenicity [3]. Recently approved adjuvants have shown promising clinical efficacy but their use is limited by toxicity (e.g., AS03), side effects or proprietary restrictions (e.g., CpG and MF59 by Pfizer and Novartis respectively). Thus, there is a need for the development of more potent and safer adjuvants or immunomodulators. Additionally, a broad range of adjuvant options will benefit at-risk or sensitive populations including pregnant women, elderly, infants, and immunocompromised individuals. For instance, AS01 used in phase 3 clinical trials of a malaria vaccine showed 95% efficacy in children 5–17 months old, but showed efficacy only in 16% of infants aged 6–12 weeks [4]. Adjuvants may show different efficacy when administered in different formulations or paired with different vaccines. Adjuvants cannot be approved as standalone formulations, but regulatory agencies will evaluate specific adjuvant/antigen formulations. This implies that the development of an adjuvant is strictly related to the antigen present in the specific formulation and may be affected by guidelines of specific regulatory agencies (e.g., FDA vs. EMA).

1.3 A cytokine-based immunomodulator as a novel molecular adjuvant.

Cytokines are known to modulate immune response via regulation of B and T cell lymphocytes, antibody production or by triggering an inflammatory response. Among cytokines, interleukins (IL) are known to regulate pathways leading to antibody production against pathogens or other antigens, including small molecules and proteins. Among interleukins, IL-4, IL-6 and IL-21 support B cell activation processes including isotype switch, affinity maturation, and clonal selection which are key events involved in the formation of long lived high affinity antibody secreting B cells and memory B cells [6]. Thus, an agent that modulates interleukin signaling can be used as a molecular adjuvant to optimize long term immune responses against challenging targets or specific patient population subsets.

1.4 Cellular events involved in antibody responses after immunization.

After immunization, the antigen binds to the B cell receptor (BCR) displayed on the surface of B cells. Binding to the BCR activates various B cell processes leading to B cell differentiation in specialized subsets, which have diverse roles in adaptive immunity. Critical to antibody production is the T cell dependent B cell activation in germinal centers (GC) of lymph nodes and spleen where antigen-specific B cells, aided by T follicular helper (Tfh) cells, differentiate into long lived antibody secreting B cells and switched immunoglobulin memory B cells [6,7].

GC formation is a complex and a poorly understood process, which is modulated by interleukins, co-stimulatory molecules, and downstream signaling pathways. Recent literature suggests that biologics or small molecules targeting these signaling pathways can be exploited to optimize humoral or cellular responses against different targets.

1.4.a. Germinal center formation

Activated B cells can differentiate along many pathways. Commitment to a specific pathway is dependent upon interleukins and co-stimulatory molecules such as ICOSL, CD40L, and CD28 [8]. Binding of some interleukins to interleukin receptors on B or T cells activates the Janus kinase/ Signal Transducer and Activator of Transcription (JAK/STAT) pathway, which control downstream transcription factors. The main pathways are outlined in the figure below:

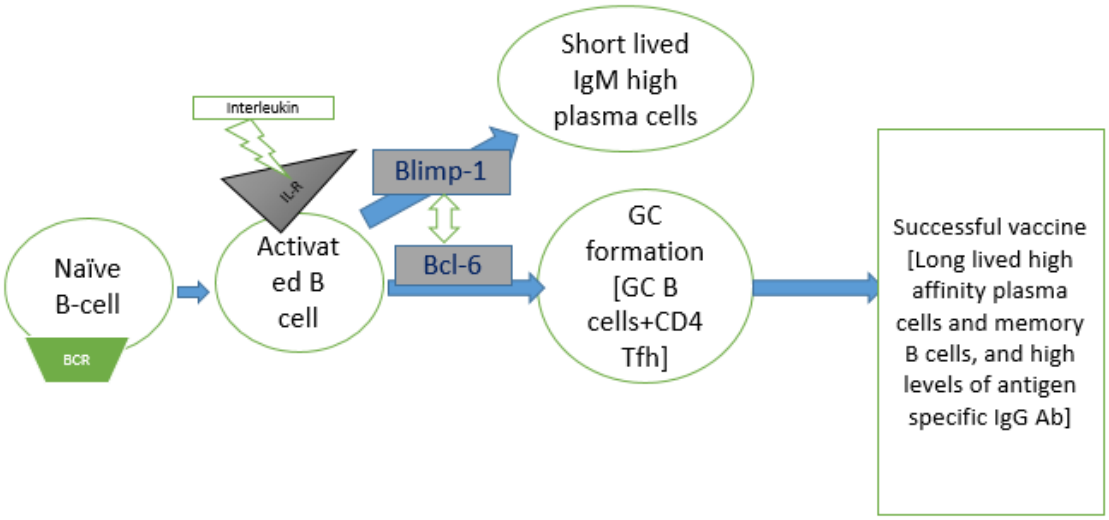


Figure 1. Major pathways of B cell activation. Activation of the B-lymphocyte-induced maturation protein (blimp-1), a transcriptional regulator is characteristic of formation of short lived antibody secreting plasma cells [9]. Activation of B-cell lymphoma 6 protein (bcl-6), a transcriptional regulator results in formation of GC B and Tfh cells [9,10,11].

A vaccine response involves binding of interleukins like IL-4 and IL-21 which leads to GC formation, isotype switch and affinity maturation via activation of the bcl-6 pathway (or down-regulation of the blimp-1 pathway) to generate long lived antigen specific antibodies [12,13]. Thus, any agent that prolongs the presence of interleukins like IL-4 induces GC formation. My project tested the hypothesis that the vaccine responses can be improved by the means of an anti-IL-4 monoclonal antibody that binds and extends the half-life of IL-4. To test this hypothesis, my studies involved the use of an adult tetanus, diphtheria, acellular pertussis vaccine (BOOSTRIX from GlaxoSmithKline).

2. Experimental Design

Male BALB/c mice (Harlan Laboratories, Madison, WI) were housed with a 12hr light /12 hr. dark cycle, and fed ad libitum. Mice were divided into 2 cohorts and immunized with TDaP plus anti-IL-4 mAb or saline. The immunization schedule was performed as shown in Table 2. In this experiment, BALB/c mice (n=14 / group) received either TDaP or TDaP plus anti-IL-4 mAb. After immunization, antigen-specific serum IgG titers were measured by ELISA using tetanus toxoid (TT) or cross-reactive material 197 (CRM_{197}) from diphtheria as coating antigens.

Table 2- Experimental protocol

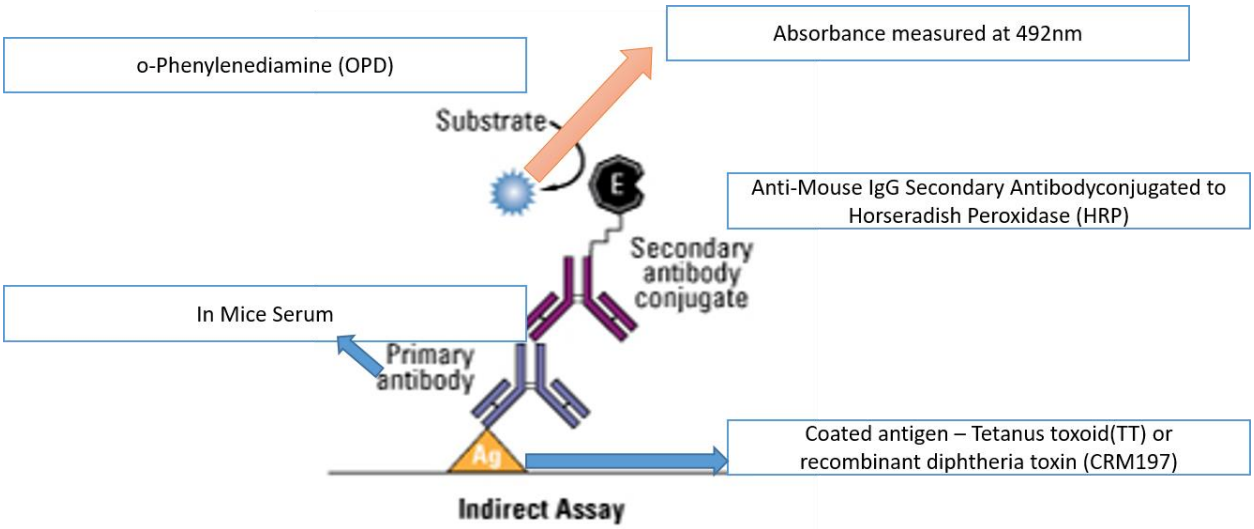
Event time	Immunization & Experimental Protocol
Day -3	Test mice were injected with an anti-IL-4 mAb (0.5mg/mice, i.p., 0.5ml injection volume). Control mice received volume-matched saline injections.
Day 0	Test and control mice were injected with TDaP vaccine (25µl, s.c.).
Day 1	Test mice were injected with an anti-IL-4 mAb (0.5mg/mice, i.p., 0.5ml injection volume). Control mice received volume-matched saline injections.
Day 14	Blood was collected by facial vein puncture to measure antibody by ELISA. Test and control mice were injected with TDaP vaccine (25µl, s.c.).
Day 28	Blood was collected by facial vein puncture to measure antibody by ELISA. Test and control mice were injected with TDaP vaccine (25µl, s.c.).
Day 42	Blood was collected by facial vein puncture to measure antibody by ELISA.

3. Materials and Methods.

3.1 ELISA for tetanus toxoid- and diphtheria-specific serum IgG antibody titers.

ELISA was performed to quantify antigen-specific IgG titers in immunized mice. To detect, TT and CRM_{197}-specific IgG titers, ELISA plates were coated with 5ng/well of TT or CRM_{197}. Chicken ovalbumin (OVA) was used as a control antigen. Coating of TT and CRM_{197} was done with carbonate buffer at pH 9.6 and blocked with 1% gelatin. Primary antibodies were incubated with anti-mouse IgG antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) to measure TT or CRM_{197}-specific serum IgG antibody titers. OPD (o-phenylenediamine dihydrochloride) was used as a water-soluble substrate for horseradish peroxidase (HRP). Absorbance was measured at 492nm.

Figure 2-Diagram of the ELISA assay



3.2 <u>Development of sensitive reagents to detect antibodies specific for TT and CRM197 in immunoassays: biotinylation.</u>

Streptavidin (SA) and avidin are proteins that bind to biotin with high affinity. Streptavidin conjugated to fluorophores, enzymes, or fluorescent proteins (e.g., phycoerythrin, PE) are used to detect biotinylated molecules in standard immunoassays such as ELISA or flow cytometry used in pre-clinical or clinical setting. During my laboratory rotation, I explored the development of conjugates consisting of antigen-biotin-SA-PE to detect antigen-specific serum IgG antibodies and B cells in mouse and human samples.

Specifically, these reagents will be used to isolate antigen-specific B cells from spleen, lymph nodes, and blood samples. In these assays, samples are first incubated with antigen-biotin-SA-PE conjugates, and then antigen-specific B cells are isolated using anti-PE magnetic beads and magnetized columns. Using flow cytometry, it is possible to further characterize the number of antigen-specific B cell populations displaying a specific phenotype. For instance, after immunization, this method allows to analyze whether a specific vaccine formulation induces memory B cells or plasma cells.

In order to develop a reliable biotinylation protocol, my efforts focused on model antigens OVA and bovine serum albumin (BSA). OVA and BSA were biotinylated using N-

hydroxysuccinimidobiotin (NHS-biotin) provided as a kit (EZ-Link™ NHS-Biotin). Tested biotinylation conditions included antigen: biotin molar ratios of 1:1, 1:2, 1:5, 1:15, 1:20, and 1:25. Antigens were dissolved in distilled water and NHS-biotin was dissolved in dimethylformamide (DMF). Biotinylation was verified using Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI TOF) by comparing the molecular weight of the native and biotinylated proteins. Samples were prepared through zip tipping using Millipore ZipTips 0.6ml C4 resin to remove salts and other small molecules, which may interfere with MALDI-TOF. The samples were hydrated using 10µl of wetting solution (1:1 acetonitrile (ACN): distilled water, 0.1% trifluoroacetic acid (TFA)) and 10µl of wash solution (0.1% TFA in distilled water). The samples were then eluted with a solution of 3:1 ACN: distilled water, 0.1%TFA. In the end, 1µl of sample and 1µl of matrix solution containing 5% sinapic acid were mixed and loaded onto the MALDI detection plate for analysis.

3.3 Statistical Analysis

Mean IgG titers were compared by unpaired T-test with 95% confidence interval by means of Graph Pad Prism 6 software.

4. Results-

4.1 Production of antigen-specific serum IgG antibodies

Serum IgG antibody titers for CRM_{197} specific IgG titers increased till day 28 and then dropped on day 42 (Figure 3B). In contrast, TT-specific IgG antibody titers showed a trend towards decrease after day 14(Figure 3A). The use of anti-IL-4 mAb as an adjuvant elicited significantly higher serum IgG titers for both tetanus toxoid and CRM_{197} on day 28 and 42. (Figure 3A & 3B).

Figure 3A- Analysis of TT-specific serum antibody titers after immunization.

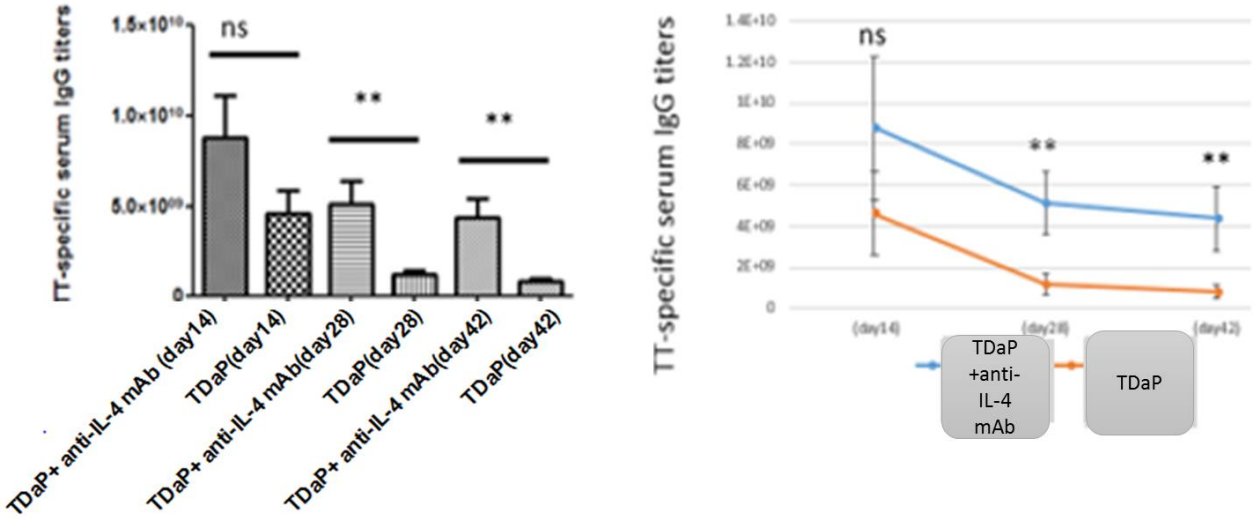
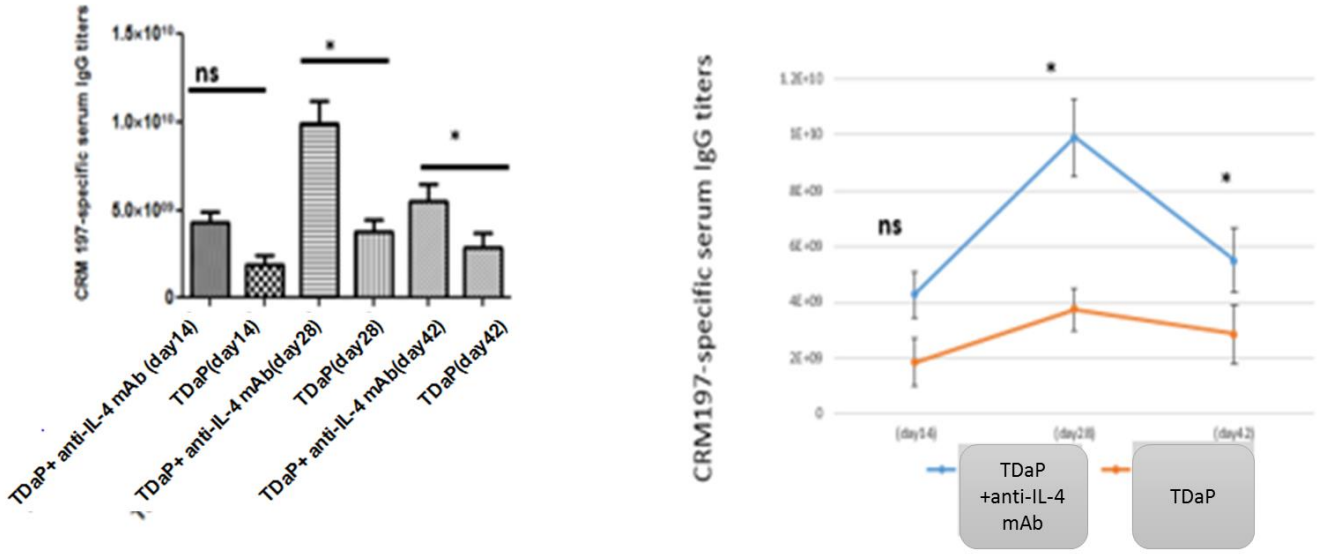


Figure 3B- Analysis of CRM_{197}-specific serum IgG antibody titers after immunization.



4.2 Biotinylation –

Haptenization ratio is the number of biotin molecules conjugated to a hapten and can be calculated by the formula-

(Molecular weight of hapten NHS conjugate) – (Molecular weight of hapten)

(Molecular weight of NHS biotin (341.38 Da))

The haptenization ratio correlated with the molar excess of biotin used in the biotinylation for haptens BSA and OVA (Figure 4B). Biotinylation of TT and CRM_{197} could not be carried out. The reason could be the unavailability of exposed amino groups in these large proteins for NHS to react and form stable amide bonds or alternatively, challenges in the detection of their molecular weight by MALDI-TOF.

Figure 4A-MALDI reports

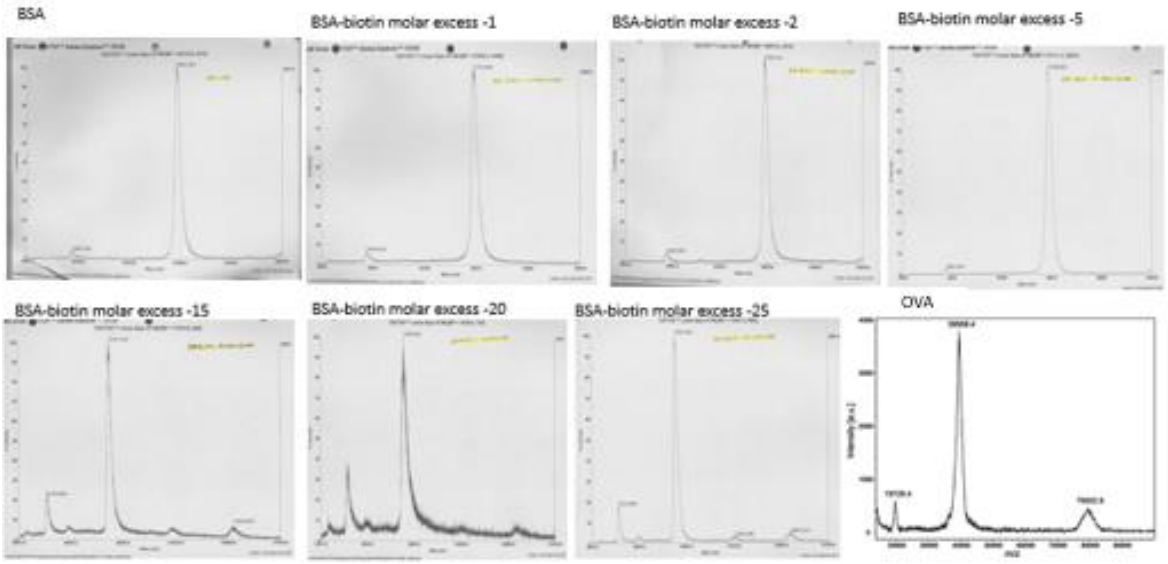
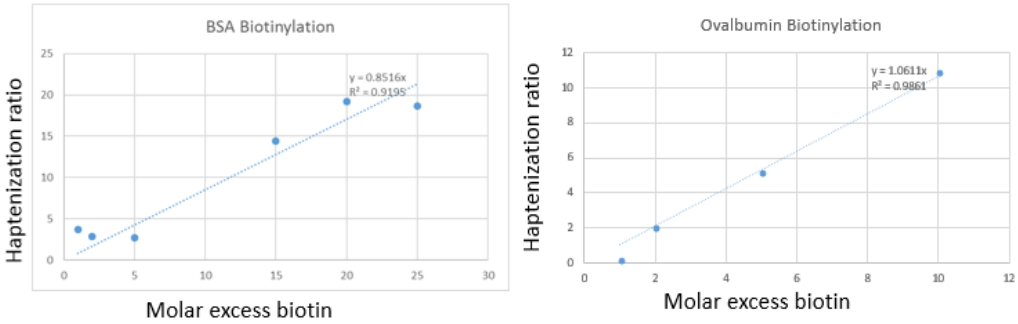


Figure 4B- Correlation between haptenization ratio and molar excess of biotin



5. Discussion and Conclusions

The importance of interleukin 4 in the antibody response of animals immunized with alum adsorbed antigen has been highlighted in several studies [14]. My results show that blocking IL-4 signaling impacts vaccine response in mice. However further studies with this monoclonal antibody are needed. If successful, the mAb could be explored for clinical use as a vaccine adjuvant in traditional and newer vaccines. The presumed mechanism of anti-IL-4 mAb is through

prolongation of IL-4 half-life by directly binding the endogenous IL-4 and slowly release it over time. Overproduction of IL-4 is associated with allergies [15]. Thus, toxicology studies should be conducted to assess the safety of this reagent.

Regulatory guidelines in the US and Europe require pre-clinical data such as proof of efficacy, pharmacokinetic parameters, and toxicity of adjuvant alone before a human trial [16,17]. Preliminary studies should establish the effect of the adjuvant on the immunological responses to the antigen(s). For instance, future studies with compounds targeting IL-4 signaling could include analysis of vaccine-specific B cells and T cell responses by means of flow cytometry, optimization of dose and determination of its pharmacokinetic parameters.

Adjuvant and formulation selection may be based on several parameters, including the physical and chemical natures of the vaccine antigen, type of immune response desired, age of the target population and route of vaccine administration. The desired qualities of each vaccine may necessitate adjuvants with specific properties. Indeed, the selection of the wrong adjuvant may render a vaccine antigen inadequate. Thus, vaccine antigen selection must consider adjuvant selection to avoid discarding potentially effective vaccine antigen candidates.

In the development of new adjuvant vaccines, it will be important to focus on clear unmet needs to establish a favorable benefit-to-risk ratio. Moreover, to engender positive public perception, rigorous clinical and post-marketing testing will be required to identify potential safety issues, as well as the mechanisms involved to guide subsequent vaccine development projects. Understanding the limitations of preclinical models will help avoid surprises in the clinic. Furthermore, understanding of the proposed mechanisms of action of existing adjuvants must continue to be refined. These aspects must play vital parts to realize all the potential benefits that adjuvants offer.

In conclusion alum-based vaccines were originally licensed more than 70 years ago. However, it remains unclear exactly how aluminum salts work as adjuvants. Moving beyond the use of alum, other approved adjuvants in human vaccines include MF59, AS03 and AS04. Future vaccines will contain recombinant protein antigens, purified synthetic adjuvants and delivery systems designed to ensure that the antigen is targeted efficiently to antigen presenting cells and that activates specific components of the adaptive immune system.

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Format –

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